Human maxillary tuberosity and jaw periosteum as sources of osteoprogenitor cells for tissue engineering

Andrea Cicconetti, MD,[a] Benedetto Sacchetti, PhD,[b] Adriano Bartoli, DDS,[c] Stefano Michienzi, PhD,[d] Alessandro Corsi, MD, PhD,[e] Alessia Funari, PhD,[f] Pamela Gehron Robey, PhD,[g] Paolo Bianco, MD,[h] and Mara Riminucci, MD, PhD,[i] Rome and L'Aquila, Italy, and Bethesda, MD
UNIVERSITÀ "LA SAPIENZA," FONDAZIONE PARCO SCIENTIFICO SAN RAFFAELE, UNIVERSITÀ DELL'AQUILA, AND NATIONAL INSTITUTES OF HEALTH

Objective. Bone tissue engineering is a promising approach for bone reconstruction in oral-maxillofacial surgery. This study investigates the suitability of oral skeletal tissues as convenient and accessible sources of osteogenic progenitors as an alternative to the iliac crest bone marrow.
Study design. Samples of maxilla tuberosity (MT) and maxillary and mandibular periosteum (MP) were obtained during routine oral surgery, and donor site morbidity was assessed using a "split-mouth" approach. Cells isolated from MT (bone marrow stromal cells; MT-BMSCs) and from MP (periosteal cells; M-PCs), were analyzed for clonogenicity, phenotype, expression of osteogenic markers, and ability to form bone in vivo.
Results. Both MT-BMSCs and M-PCs included clonogenic cells, showed comparable phenotypic profiles, and expressed early osteogenic markers. Most importantly, both cell populations formed bone upon ectopic in vivo transplantation.
Conclusion. MT-BMSCs and M-PCs behaved as osteoprogenitor cells in vitro and in vivo. MT and MP may be considered as suitable sources of cells for bone tissue engineering in humans. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;xx:1-12)**

Allogeneic bone and alloplastic bone substitutes are successfully used as void fillers and osteoconductive agents in oral-maxillofacial surgery[1]; however, serious concerns still exist about the high rate of graft resorption, limited regeneration by new bone, hazards of immune-mediated rejection, and donor-host pathogen transfer.[2-5] Infection and failure of the graft, severe morbidity of extraoral donor sites, and an unpredictable cost-benefit ratio are the main problems associated with maxillary reconstructive techniques, such as guided bone regeneration, sinus lift, and onlay grafts.[6-10] Autogenous bone is the gold standard in bone reconstructive surgery. Compared to bone substitutes, autogenous bone has superior biomechanical properties and greater potential for adequate integration with surrounding tissues; however, eventual loss of the grafted bone tissue stands as a major untoward outcome of autogenous bone grafting. The mechanism underlying the progressive loss of grafted bone has not been fully elucidated, but may be related to the limited survival of bone cells after transplantation[11,12] or to the relatively low frequency of self-renewing progenitor cells within the graft. The different embryonic origins of the splanchnocranial skeleton (neuroectoderm) and the axial/appendicular skeleton (mesoderm) could also play a role in the frequent resorption of cranio-maxillo-facial bone grafts[13] obtained from extracranial sites.

Bone tissue engineering, through the use of cell-biomaterial constructs, is emerging as a promising approach to the regeneration of bone in a variety of clinical settings. The approach is based on the use of

This study was supported by grants from (Ministero dell'Istruzione dell'Università e della Ricerca), AIRC (Associazione Italiana per la Ricerca sul Cancro), and the European Union (GENOSTEM) to P.B.
[a]Assistant Professor, Departamento di Scienze Odontostomatologiche, Universitá "La Sapienze."
[b]Post-Doctoral Fellow, Fondazione Parco Scientifico San Raffaele.
[c]Free Attendant, Dipartamento di Scienze Odontostomatologiche, Universitá "La Sapienze."
[d]Post-Doctoral Fellow, Fondazione Parco Scientifico San Raffaele.
[e]Assistant Professor, Dipartimento di Medicina Sperimentale e Patologia, Universitá "La Sapienze."
[f]Post-doctoral Fellow, Fondazione Parco Scientifico San Raffaele; and Dipartimento di Medicina Sperimental Universitá dell'Aquila.
[g]Branch Chief, Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Departments of Health and Human Services.
[h]Full Professor, Fondazione Parco Scientifico San Raffaele; and Dipartimento di Medicina Sperimentale e Patologia, Universitá "La Sapienze."
[i]Associate Professor, Fondazione Parco Scientifico San Raffaele; and Dipartimento di Medicina Sperimental Universitá dell'Aquila.
Received for publication Aug 11, 2006; returned for revision Dec 30, 2006; accepted for publication Feb 15, 2007.
1079-2104/$ - see front matter

doi:10.1016/j.tripleo.2007.02.022

osteoconductive materials and autologous cells endowed with inherent osteogenic potential and broad regeneration capacity. The recognition of the existence and properties of skeletal (mesenchymal) stem cells in bone marrow has opened important new perspectives for bone tissue engineering at large[14,15] and for reconstruction of gnathic bones in particular.[16] Endowed with significant growth potential and native osteogenic capacity, skeletal stem cells can be isolated from the bone marrow stroma, expanded in culture, and then transplanted in vivo to the effect of generating significant amounts of structurally normal bone.[17-21] As applied to preclinical in vivo models, this general approach has shown distinct potential for the filling of bone defects either in limbs or in craniofacial bones.[22-24] Pilot clinical experiences have further validated the principles underlying the use of cell populations that contain skeletal progenitor cells for bone regeneration.[25-27] Protocols for the isolation and culture of osteogenic progenitor cells, and the choice and design of the relevant osteoconductive carrier, represent the most important open questions in bone tissue engineering.[15] Procedures and tools that are specific for each particular clinical application need to be designed through relevant preclinical studies, addressing the tissue source of progenitor cells, culture procedures, choice of material, and surgical procedure. Skeletal (mesenchymal) stem cells are typically within cell populations isolated from iliac crest bone marrow[19]; however, it has been claimed that osteogenic progenitor cells can also be isolated from additional anatomical sites or tissues, in particular, from adipose tissue.[28] Nonetheless, the inherent osteogenic ability of cell strains obtained from tissues other than bone marrow or from skeletal sites other than the iliac crest, has seldom been validated in comparison with bone marrow stromal cells through the use of appropriate assays. In particular, in vivo transplantation assays remain the gold standard by which to assess the ability of a given cell strain to form bone in vivo, and therefore to validate in a preclinical setting the use of specific types of cell populations for clinical use.

This study aims to validate the potential of 2 intraoral sites, the maxillary tuberosity and the maxillary/mandibular periosteum, as sources of osteoprogenitor cells for future applications in oral-maxillofacial bone tissue engineering. Cells isolated from the 2 skeletal compartments were compared for their clonogenic efficiency, phenotype, and in vivo osteogenic potential. Furthermore, signs of postoperative morbidity related to the harvest of tissue from these 2 intraoral sites were evaluated in order to develop a feasible procedure for future applications in oral surgery.

MATERIALS AND METHODS

Patients

Seven maxillary tuberosity (MT) and 7 maxillary or mandibular periosteal (MP) samples were harvested from 7 patients, 3 males and 4 females, aged 19 to 32 years, who underwent routine oral surgery procedures (removal of bilateral impacted teeth and/or bilateral odontogenic cysts). Patients with metabolic bone diseases, local inflammatory disease, or impaired blood coagulation were excluded from this study, as were patients on medication affecting the skeletal metabolism (e.g., corticosteroids) and pregnant women. The study was performed under an institutionally approved protocol and all patients gave written informed consent.

Harvests of MT and MP tissues

Under general anesthesia, a full-thickness MP biopsy and a standardized quantity of bone from the MT were harvested concomitantly with the removal of bilateral impacted teeth/odontogenic cysts. We used a "split-mouth" method to compare the difference in postoperative morbidity between the site in which tooth/cyst removal was associated with MT and MP tissue harvesting (study site) and the contralateral site in which oral surgery alone was performed (control site). At the study site, a full-thickness flap was generated by using a blunt periosteal elevator to reach the impacted tooth or odontogenic cyst, without damaging the "osteogenic" inner layer of the periosteum. Care was exercised to avoid overextension of the peeled flap, which could cause subsequent swelling. An intact periosteal sheet, measuring 5×5 mm, separated from the underlying bone, was easily peeled off using a scalpel blade (Fig. 1, *A*). Then, the removal of the embedded tooth/odontogenic cyst was performed, and a 1-cm^3-sized MT bone sample was harvested using a U-shaped chisel (Fig. 1, *B*). The harvested tissues were immediately transferred to the laboratory under sterile conditions. Postoperative pain intensity and duration, edema, and hemorrhage were compared between study and control sites.

Cell culture

Maxillary tuberosity-bone marrow stromal cell (MT-BMSC) culture. Under sterile conditions, the MT samples were scraped gently in α-modified Minimum Essential Medium (αMEM; Invitrogen Life Technologies, Carlsbad, CA) using a steel blade and washed repeatedly to release marrow cells. The marrow cell suspensions were passed through an 18-gauge needle to break up cell aggregates and filtered through a 70-μm pore-size cell strainer (Becton Dickinson Labware, San Diego, CA) to obtain a single cell suspension. Bone marrow nucleated cells

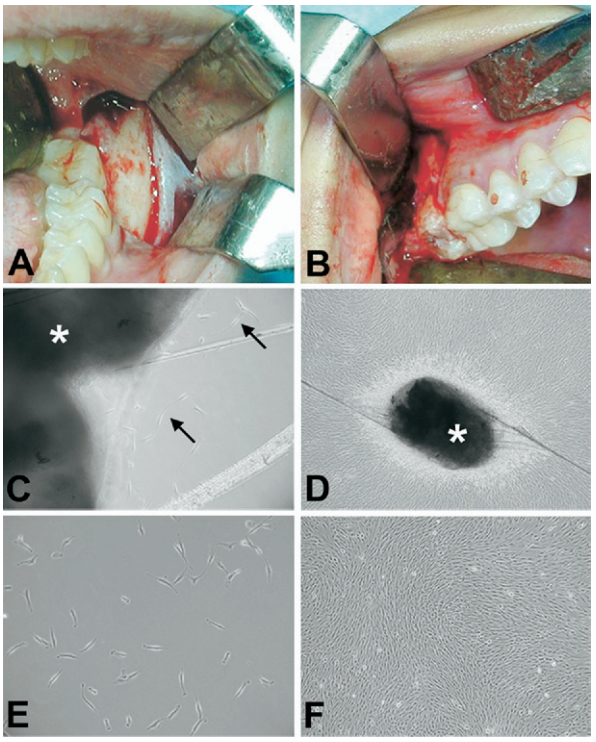

Fig. 1. (**A, B**) Harvesting of surgical samples. A full-thickness flap was elevated and a maxillary/mandibular periosteum sheet was peeled off by using a scalpel blade (**A**). After removal of the impacted tooth/odontogenic cyst, a maxillary tuberosity bone sample was harvested by using a U-shaped chisel (**B**). (**C-F**) Cell cultures established from fresh maxillary/mandibular tissues. Periosteal cells were grown from tissue explants (**C**) and reached confluence in 2 weeks (**D**). Maxillary marrow stromal cells were isolated by adherence from marrow cell suspensions (**E**) and generated a cell layer after 2 weeks in culture (**F**) (*arrows,* periosteal cells; *asterisks,* periosteal tissue explants). (**A–F**, original magnification ×200.)

(BMNCs) were then plated at high density (5×10^6 cells) in 150-mm culture dishes in a growth medium consisting of αMEM (Invitrogen Life Technologies), 20% lot-selected fetal bovine serum (FBS; Invitrogen Life Technologies), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. In some cultures, the medium was supplemented with 10^{-8} M dexamethasone (Sigma, St. Louis, MO) and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (Sigma). Cultures were incubated at 37°C in a 5% CO_2 atmosphere. On the third day, nonadherent (hematopoietic) cells were removed by washing the cultures with calcium/magnesium-free phosphate-buffered saline (PBS; Invitrogen Life Technologies); adherent cells were fed twice weekly thereafter. At confluence, cells were washed twice with Hank's Balanced Salt Solution (HBSS), released from the substrate using a 0.25% trypsin/EDTA solution (Invitrogen Life Technologies) and plated again for further studies.[29]

Maxillary/mandibular periosteal cell (M-PC) culture. Periosteal cells were grown from tissue explants as adherent cells. The periosteal sheets were washed twice using HBSS and then manually minced with scissors. Tissue fragments were then placed into 150-mm culture dishes containing complete medium (αMEM, 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin), which was replaced every third day. When grown to subconfluence, adherent cells were released by trypsin and plated again for further characterizations as described below. Tissue fragments were discarded.

Proliferation activity

Proliferation activity was evaluated from the first to the fourth passage in vitro. At passage 1 (P1), 5×10^3 cells/cm^2 were plated in 10-mm Petri dishes, the number of population doublings (PD) and the doubling time (DT) were then assessed at P2, P3, and P4 as follows: (PD) = Log (cells counted/cells plated) × 3.33; (DT) = days in culture/PD.

Clonogenicity assay

For bone marrow samples from the maxillary bone, clonogenicity assays were conducted essentially as established for iliac crest bone marrow samples.[18,29-31] Briefly, single cell suspensions of total bone marrow nucleated cells (BMNCs) were plated at a density of 6×10^3 cell/cm^2. To conduct clonogenicity assays for periosteal tissue, periosteal samples were enzymatically digested to generate a single cell suspension. Samples were digested with 100 U/mL *Clostridium histolyticum* collagenase type II (Invitrogen Life Technologies) in HBSS, 3 mM $CaCl_2$. Cells released from 2 sequential digestions were pooled and used for clonogenicity assays. Since cell suspensions generated by enzymatic digestion of periosteal samples do not include substantial numbers of nonadherent hematopoietic cells (at variance with marrow samples), periosteal cells were plated at densities of 1.6 to 1.6×10^2 cells/cm^2. After 2 weeks, cultures were fixed with 4% formaldehyde, stained with Giemsa stain (Sigma) for 30 minutes at room temperature, and colonies (>50 cells) were counted. The clonogenic activity of each population was expressed as number of colonies/number of plated cells in the case of periosteal cells. For bone marrow stromal cells, it was expressed as colony-forming efficiency (CFE) normalized to 10^5 BMNCs as per established methods.[32]

Table I. Antibodies used for fluorescence-activated cell sorting (FACS) analysis

Antibody	*Clone*	*Labeling*	*Source*
CD105	Clone 266	FITC	Becton Dickinson-Pharmingen (San Diego, CA)
CD 49a	Clone SR84	FITC	Becton Dickinson-Pharmingen
CD 63	Clone H5C6	PE	Becton Dickinson-Pharmingen
CD 90	Clone 5E10	CY-C	Becton Dickinson-Pharmingen
CD 140b	Clone 28D4	FITC	Becton Dickinson-Pharmingen
ALP	Clone B4-78	FITC	R&D Systems Inc (Minneapolis, MN)
CD 61	Clone VIPL2	FITC	Becton Dickinson-Pharmingen
CD 71	Clone M-A712	PE-Cy5	Becton Dickinson-Pharmingen
CD 45	Clone HI30	FITC	Becton Dickinson-Pharmingen
CD 133	Clone 170411	FITC	R&D Systems
CD 117	Clone YB5B8	CY-C	Becton Dickinson-Pharmingen
CD 34	Clone 581	FITC	Becton Dickinson-Pharmingen

ALP, alkaline phosphatase; *CY-C,* CyChrome; *Cy-5,* CyChrome5; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin.

Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry analysis was performed on cells grown to passage 3 in standard medium by using the panel of monoclonal antibodies reported in Table I. After release from the substrate, cells were centrifuged at 190 g for 10 minutes and resuspended in blocking buffer (HBSS supplemented with 30 mM HEPES, 5% FBS, and 0.1% sodium azide, pH 7.3) for 20 to 30 minutes at 4°C on ice; 10^5 cells were used for each marker. After incubation with each antibody for 30 minutes at 4°C on ice, cells were washed and resuspended in PBS/FBS. Analysis of cell surface molecules was then performed by using a FACSCalibur flow cytometer (Becton Dickinson Labware). Ten thousand events were recorded for each sample and data were analyzed with the CellQuest software (Becton Dickinson Labware).

Expression of osteogenic markers

Expression of osteogenic transcription factors, Runx2/Cbfa1 and Osterix, and bone matrix proteins, bone sialoprotein (BSP) and osteocalcin (OC), was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR (q-PCR). Total RNA was isolated from confluent cultures using Trizol (Sigma), and cDNAs were generated using random primers and the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies), both according to the manufacturers' instructions. mRNA from human trabecular bone cells (HTBCs) and human bone marrow stromal cells (BMSCs) established as described previously[33,18] were available from separate studies and used as controls.

For RT-PCR the following primers were used:

- Runx2: forward 5′-GAG GGT ACA AGT TCT ATC TGA A-3′; reverse 5′-GGC TCA CGT CGC TCA TTT TG-3′; amplified fragment size 200 base pairs (bp) (GenBank Accession Number NM_001015051).
- Osterix: forward 5′-GCA GCT AGA AGG GAG TGG TG-3′; reverse 5′-GCA GGC AGG TGA ACT TCT TC-3′; amplified fragment size 359 bp (GenBank Accession Number AF477981).
- Bone sialoprotein: forward 5′-CCA ATG CAG AAG ACA CCA CAG-3′; reverse 5′-AGG CCC TGG TGG TGG TAG TA-3′; amplified fragment size 330 bp (GenBank Accession Number BC111920).
- Osteocalcin: forward 5′-GCC GTA GAA GCG CCG ATA GGC-3′; reverse 5′-ATG AGA GCC CTC ACA CTC CTC-3′; amplified fragment size 259 bp (GenBank Accession Number BC113434).
- GAPDH: forward 5′-CGG GAA GCT TGT GAT CAA TGG-3′; reverse: 5′-GGC AGT GAT GGC ATG GAC TG-3′; amplified fragment size 358 bp (GenBank Accession Number M17851).

The target cDNA sequences were amplified in standard PCR reactions using 2.5 units of Platinum Taq polymerase. After a denaturation step at 94°C for 5 minutes, the reactions were run for 33 cycles at the following temperatures: 94°C, 45 seconds; 49°C (BSP, OC) or 55°C (GAPDH) or 58°C (Runx2, Osterix), 45 seconds; 72°C 45 seconds, with a final extension at 72°C for 5 minutes.

For quantitative RT-PCR, cDNAs were amplified by using gene-specific Taqman oligonucleotides (Applied Biosystem, Foster City, CA) according to the manufacturer's instructions and the ABI PRISM 7000 Sequence Detection System (Applied Biosystem). Delta-CT values were normalized based on amplification of GAPDH and the results were expressed as fold expression relative to values obtained for HTBCs.

Alkaline phosphatase (ALP) cytochemistry

MT-BMSCs and M-PCs were grown in standard medium and in medium supplemented with 10^{-8} M dexamethasone and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (Sigma). After 2 weeks in culture, cells were washed twice with PBS, fixed with 4% formaldehyde in phosphate buffer, pH 7.4, and reacted for alkaline phosphatase using Naphtol AS phosphate as substrate and Fast Blue BB as coupler. Naphtol AS phosphate was dissolved in N-N' dimethylformamide (30 mg in 0.5 mL) and added to a 0.1% solution of Fast Blue BB salt in 0.1% boric acid/sodium tetraborate buffer, pH 9. Cultures were incubated in the ALP substrate solution for 20 minutes at 37°C.

In vivo transplantation

MT-BMSC and M-PC cultures were expanded in vitro in the presence of 10^{-8} M dexamethasone and 10^{-4} ascorbic acid. At confluency, cells were released by trypsin, centrifuged at 1000 rpm for 10 minutes and loaded onto particles (100 to 200 mm in size) of hydroxyapatite/tricalcium phosphate (HA/TCP; Zimmer, Warsaw, IN). For each transplant, 3×10^6 cells were resuspended in 1 mL of fresh medium and incubated with 40 mg of HA/TCP for 2 hours at room temperature with slow rotation. After incubation, samples were briefly centrifuged, the medium was removed and 30 mL of human fibrinogen (Sigma) and 30 mL of human thrombin (100 U/mL [Sigma]) were added to each cell-carrier construct. Adhesion of cells to the carrier was evaluated by counting the cells left in the incubation medium. Adherent cell numbers in the presence or absence of dexamethasone were statistically compared by analysis of variance (ANOVA). In vivo transplantation was performed essentially as described.[17,18] Constructs were transplanted subcutaneously into the back of immuncompromised (nih/nu/xid/bg) mice (Charles River Laboratories, Wilmington, MA) under an institutionally approved protocol for the use and care of animals in research. Transplants of cell-free HA/TCP particles served as controls. All transplants were harvested 8 weeks after surgery and analyzed histologically. Samples were fixed with 4% formaldehyde, decalcified by a neutral solution of EDTA, and embedded in paraffin. Sections, 5-μm thick, were cut from each block and stained with hematoxylin and eosin.

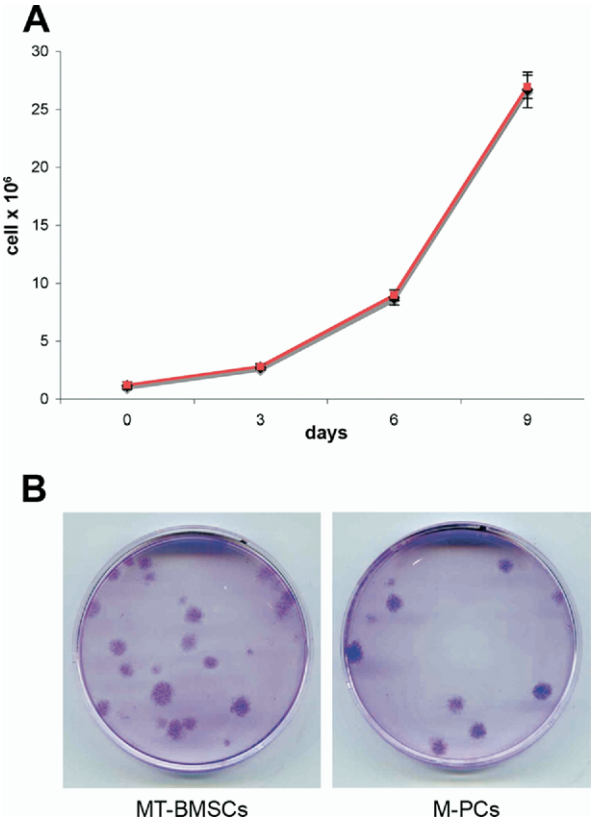

Fig. 2. (**A**) Ex vivo expansion of human maxillary tuberosity–bone marrow stromal cells (*black line*) and maxillary/mandibular periosteal cells (*red line*) revealed a comparable rate of growth. (**B**) Clonogenic cells were isolated from either the M-PC or the MT-BMSC (Giemsa stain).

RESULTS

Patient outcomes

No significant morbidity was observed or reported by patients at any of the tissue harvest sites as compared to contralateral control sites. The preoperative full-mouth disinfection protocol and immediate transfer of the tissues into sterile HBSS was effective in avoiding contact-contamination of the samples and potential risk of impairment of the primary cell cultures for growth of fungal colonies or other microorganisms.

Isolation of cells from human periosteum and maxillary bone marrow

Cells were isolated in vitro from both MP and MT samples. M-PCs grew from tissue explants starting from the fourth or fifth day of culture (Fig. 1, *C, arrows*). In MT-BMSC cultures, adherent cells were detected a few hours after plating of marrow cell suspensions (Fig. 1, *E*). In both types of cultures, cells exhibited an elongated, fibroblast-like morphology and reached confluence in 2 weeks (Fig. 1, *D* and *F*).

Ex vivo proliferation of M-PCs and MT-BMSCs

Because of the different methods applied for establishing M-PC cultures (from tissue explants) and MT-BMSC cultures (from cell suspensions), the proliferation activity of the 2 types of cultures was assessed and compared starting from the first passage in vitro, when a defined number of cells could be plated for each sample. From P1 to P4, cell counting revealed an equivalent rate of increase in the cell number in all cultures (Fig. 2, *A*). Accordingly, the number of PD and the DT were comparable in M-PC and MT-BMSC cells with the following mean values: PD 4.52 ± 0.39 and 4.6 ±0.3 for M-PCs and MT-BMSCs, respectively; DT of 1.9 ± 0.17 days and 1.9 ± 0.13 days for M-PCs and MT-BMSCs, respectively.

M-PCs and MT-BMSCs include a subset of clonogenic cells

To analyze the presence and the frequency of adherent clonogenic cells (colony forming unit-fibroblasts, CFU-Fs), clonogenic cultures were established by plat-

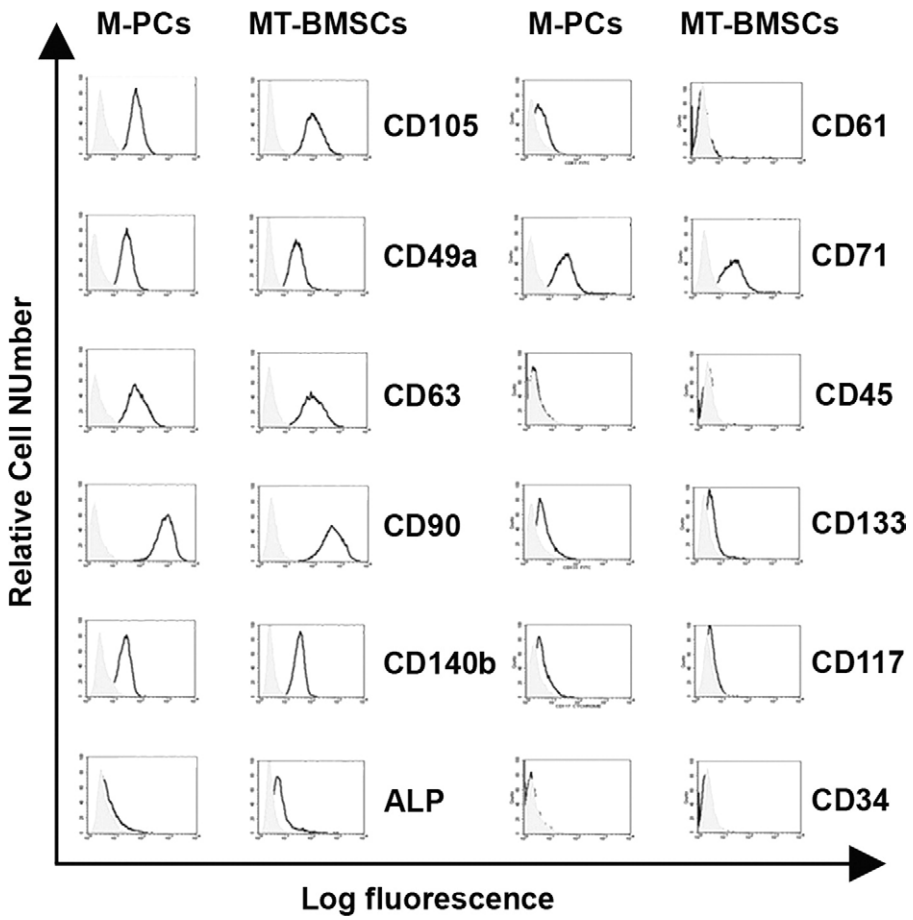

Fig. 3. Flow-cytometry analysis of human M-PCs and MT-BMSCs grown in vitro to the third passage. The 2 cell populations showed a similar surface phenotype.

ing cells at low densities. After 2 weeks in culture, discrete colonies were observed in all samples (Fig. 2, *B*), revealing a CFU-F frequency of $4.0 \pm 4.5/10^2$ cells for periosteal samples at the lowest density of plating and $10.0 \pm 2.3/10^5$ cells for maxillary marrow stromal populations.

Cell surface phenotype of M-PCs and MT-BMSCs

Analysis of the surface molecular profile of M-PCs and MT-BMSCs revealed an overall similar phenotype in the 2 populations. CD34 (a marker of endothelial cells) and CD45 were consistently negative. High levels of expression of multiple putative markers of "mesenchymal stem cells" (CD105, CD49a, CD63, CD90, CD140b) were observed in both populations. CD71 (transferrin receptor, a marker of proliferating cells)[34,35] was also highly expressed in both populations, whereas ALP was expressed at low levels in both populations. Very low levels of expression of CD61, CD133, and CD117 were variably observed in M-PCs but not in MT-BMSCs (Fig. 3).

M-PCs and MT-BMSCs express early osteogenic markers

Expression of osteogenic markers was assessed by standard RT-PCR (Fig. 4, *A*) and q-PCR (Fig. 4, *B*) experiments in which iliac crest (IC)-BMSCs and HTBCs were included for comparison as progenitors and fully differentiated osteogenic cells respectively. Expression of the transcription factor, Runx2, the earliest marker of the osteogenic lineage, was consistently detected in both M-PCs and MT-BMSCs, although at a lower level as compared to IC-BMSCs and HTBCs. Osterix, a transcription factor downstream of Runx2 that is highly expressed in mature osteoblastic cells (HTBCs) but not in osteogenic progenitors (IC-BMSCs), was undetectable or expressed at very low levels in M-PCs and MT-BMSCs. Accordingly, no expression of the bone matrix proteins, BSP and OC, which are produced by differentiated osteogenic cells, was detected either in M-PCs or in MT-BMSCs.

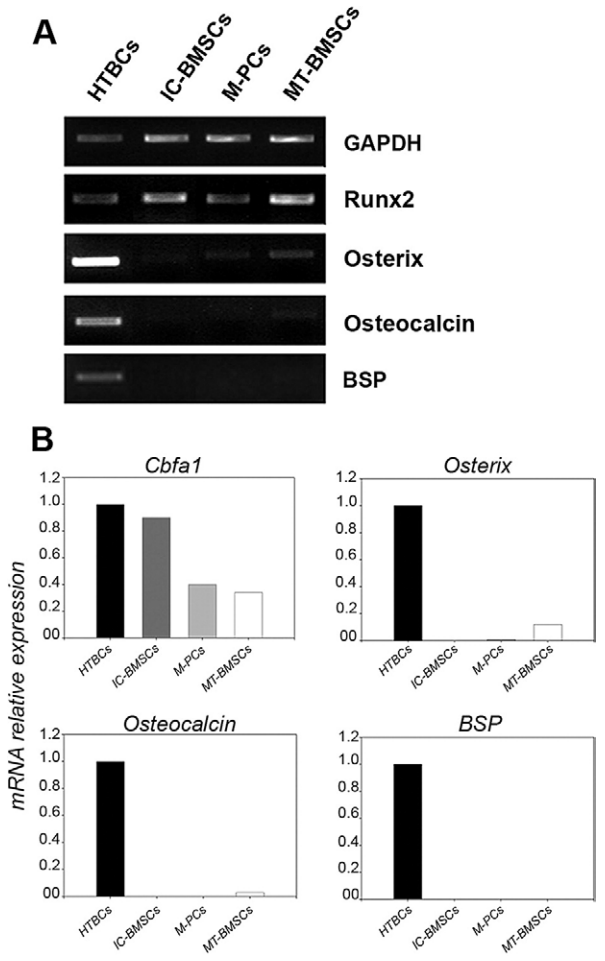

Fig. 4. (**A**) Expression of osteogenic markers in MT-BMSCs, M-PCs, IC-BMSCs, and differentiated HTBCs as detected by RT-PCR. In analogy with IC-BMSCs, M-PCs and MT-BMSCs expressed the transcription factor Runx2 and Osterix, and were negative for later markers of osteogenic differentiation. (**B**) Quantitative analysis of Runx2, Osterix, Osteocalcin, and BSP mRNAs was performed by real time PCR and reported as fold expression relative to values obtained for HTB cells.

Response of M-PCs and MT-BMSCs to factors stimulating osteogenic differentiation

We then analyzed the ability of M-PC and MT-BMSC progenitors to respond to factors such as dexamethasone, known to stimulate osteogenic differentiation of marrow stromal cells. Cultures were grown either in standard medium or in medium supplemented with 10^{-8} M dexamethasone and 10^{-4} M ascorbic acid, and expression of the enzyme, ALP, which increases during osteogenic differentiation, was analyzed by cytochemistry and FACS. In standard medium (basal condition), low levels of enzymatic activity (Fig. 5, *A, left panels*) and low levels of expression of the molecule on cell surface (Fig. 5, *B, left panels*) were detected. However, upon stimulation with dexamethasone and ascorbic acid, a marked increase in ALP expression was observed by both methods in all MT-BMSC and M-PC cultures (Fig. 5, *A* and *B, right panels*).

M-PCs and MT-BMSCs generate bone tissue in vivo

The ability of M-PC and MT-BMSC progenitors to differentiate and generate skeletal tissues in vivo was investigated by using a model of xenogeneic transplantation in the immunodeficient mouse. Cells were expanded ex vivo either in standard growth medium or in medium supplemented with dexamethasone and ascorbic acid, and then loaded onto particles of HA/TCP. Before transplantation, the number of cells that adhered to the carrier was evaluated for each construct by counting the cells left in the incubation medium. This revealed that culture conditions strongly influenced the ability of both M-PCs and MT-BMSCs to attach to carrier particles. The number of attached cells was significantly lower (15%-25%) when cells were grown in standard medium as compared to cultures expanded and loaded in the presence of dexamethasone (90%-100%) (Fig. 6). Based on these results, only the latter samples were used in the in vivo assay and 16 transplants (8 with M-PCs and 8 with MT-BMSCs) were performed. Eight weeks after transplantation, newly formed bone was observed in all samples (Fig. 7). The bone tissue, deposited onto carrier surfaces, was predominantly lamellar in structure. Fully differentiated osteocytes could be clearly recognized within the bone matrix, thus indicating the ability of transplanted cells to undergo complete osteogenic differentiation (Fig. 7, *C* and *F, arrows*). No bone formed in control transplants of cell-free carrier.

DISCUSSION

In an attempt to identify easily accessible sources of cell populations specifically suitable for facial bone regeneration, we have isolated osteoprogenitor cells from 2 distinct anatomical compartments of jawbones, the periosteum and the bone marrow. As a first step toward the identification of osteoprogenitor cells, we sought to demonstrate the presence of clonogenic cells in both compartments. Clonogenicity, i.e., the ability of a single cell to form a colony in vitro after plating a single cell suspension at low density, reflects the ability of individual cells to grow in a density-insensitive manner, indicative of a high proliferation capacity, and a characteristic of progenitor cells.[36] It is well established that in the iliac crest bone marrow, the canonical site from which osteogenic cells are isolated, skeletal stem cells and osteogenic precursors are in fact comprised in the clonogenic fraction.[18,20,37,38] Given the

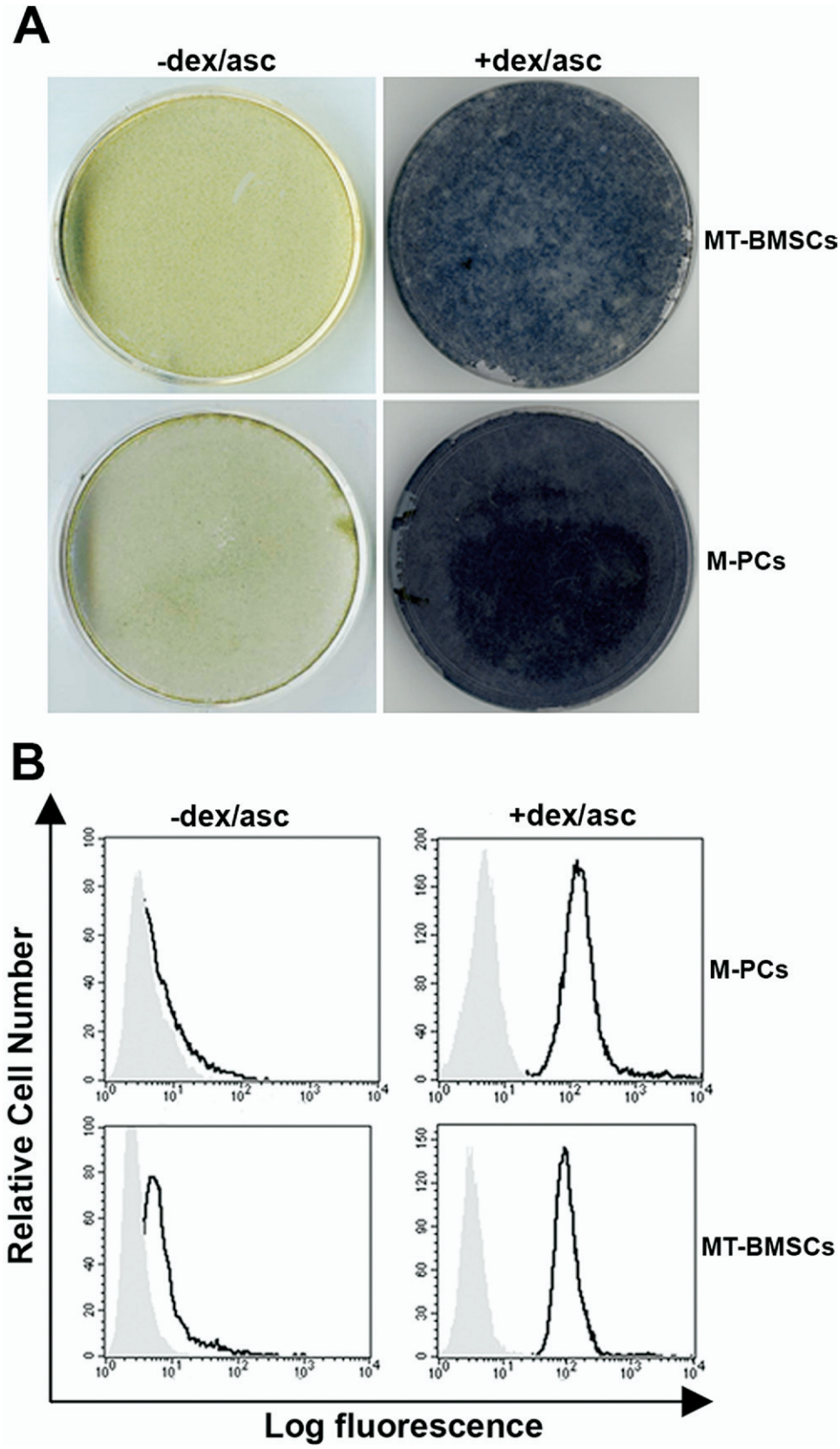

Fig. 5. Alkaline phosphatase expression as assessed by cytochemical reaction (**A**) and flow cytometry (**B**) in MT-BMSCs and M-PCs. High levels of expression of the enzyme are detected in both cell types upon stimulation with 10^{-8} M dexamethasone and 10^{-4} M ascorbic acid (**A** and **B**, *left panels*).

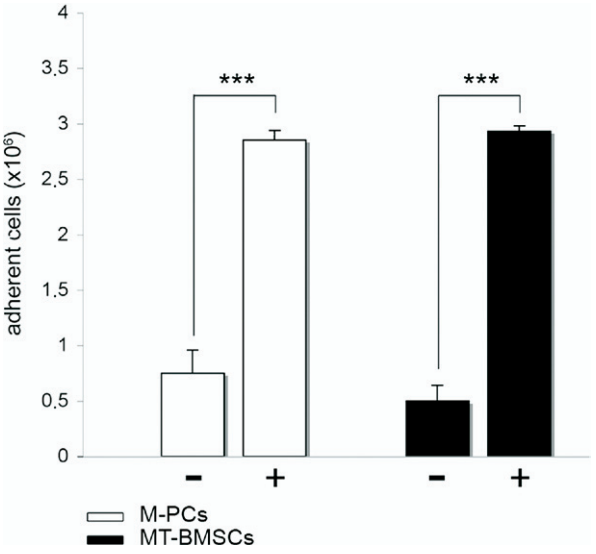

Fig. 6. Adhesion of MT-BMSCs and M-PCs to HA/TCP in the absence (–) and in the presence (+) of dexamethasone. Data are expressed as mean ± SD (n = 5). Adhesion of cells to HA/TCP was significantly increased (ANOVA; ***$P < .001$) and approached 100% when cells were grown and incubated with the carrier in the presence of dexamethasone.

distinct embryological origin of craniofacial bones, their unique anatomical features, and their distinct growth and remodeling patterns, the efficient isolation of osteoprogenitor cells from craniofacial bones is not automatically predicted by observations made on bones of the axial and appendicular skeleton. To date, only 2 studies have addressed this issue revealing some differences in the phenotypic and functional features of orofacial and iliac crest–derived stromal cells.[39,40] In this study, cell cultures from the maxillary tuberosity were established by using the same procedure used for the iliac crest, which allows isolation of bone marrow stromal cells without releasing cells from the interior of bone trabeculae.[31,33] Although a direct comparison of marrow samples obtained from the maxilla and the iliac crest of the same patients was not feasible in our study, our data show that the frequency of clonogenic cells in the maxillary bone, assessed as CFE, is directly comparable to the one observed in the iliac crest bone marrow in other studies. In agreement with the findings of Akintoye et al.[39] and Matsubara et al.,[40] who studied alveolar bone aspirates, our data also demonstrate that cells from maxillary bone are clearly endowed with in vivo osteogenic capacity.

Whereas the periosteum is notoriously associated with an osteogenic capacity (dramatically highlighted by the contribution of the periosteum to fracture healing), whether this property reflects the presence of true stem cells in the periosteum, similar to those found in the bone marrow, rather than more restricted osteogenic progenitors, has not yet been elucidated. Traditionally, periosteal cell strains are generated by explant culture procedures, in which cells migrate out of fragments of whole periosteal tissues that are placed in culture as such. Per se, this procedure prevents the identification of clonogenic cells. Although not unique of multipotent stem cells, clonogenicity is a recognized property of progenitor cells.[31] In the iliac crest bone marrow, it is generally assumed that both multipotent stem cells and committed osteogenic progenitors are comprised within the clonogenic subset of stromal cells, although distinguishing one type of progenitor from the other has remained difficult.[31] It was only recently that clonogenic cells were isolated from the periosteum of the human tibia.[41] We have now shown that, in maxillofacial bones, clonogenic cells are found in the maxillary/mandibular periosteum, in addition to the maxillary bone. To demonstrate clonogenic cells in the periosteum, however, the collagen-rich periosteal tissue needs to be enzymatically digested, and the resultant cell suspension plated in culture at clonal density (<1.6 cells/cm^2). As applied to our samples, the latter approach revealed that clonogenic cells are indeed present in the maxillary/mandibular periosteum, and indeed are highly frequent ($\sim$4%). A crude comparison of the CFE observed for periosteal cell suspensions in our study with the CFE reported for bone marrow cell suspensions[30] would predict a frequency of clonogenic cells much higher in the periosteum than in the bone marrow. However, considering that hematopoietic tissue is present in the bone marrow but not in the periosteum, a direct comparison of the frequency of CFU-F in the periosteum and bone marrow is not straightforward, as a much higher relative proportion of adherent, nonhematopoietic cells is included in the periosteum as in any connective tissue other than the bone marrow. Likewise, a more precise definition of the properties of clonogenic cells within the maxillary tuberosity and the maxillary and mandibular periosteum, and the potential presence of multipotent cells among them, will require further study. For applicative purposes, however, it is important to note that osteogenic progenitors (whether differentiation-restricted or multipotent) do exist within, and can be isolated from, the gnathic bones.

When analyzed in terms of cell morphology and proliferation in culture, cell strains obtained from either the maxillary tuberosity or the periosteum of maxillary/mandibular bone seem remarkably similar to one another. In addition, an analysis of their surface phenotype conducted using antibodies recognizing multiple putative markers of "mesenchymal stem cells" revealed a virtually identical profile. Like the bone marrow

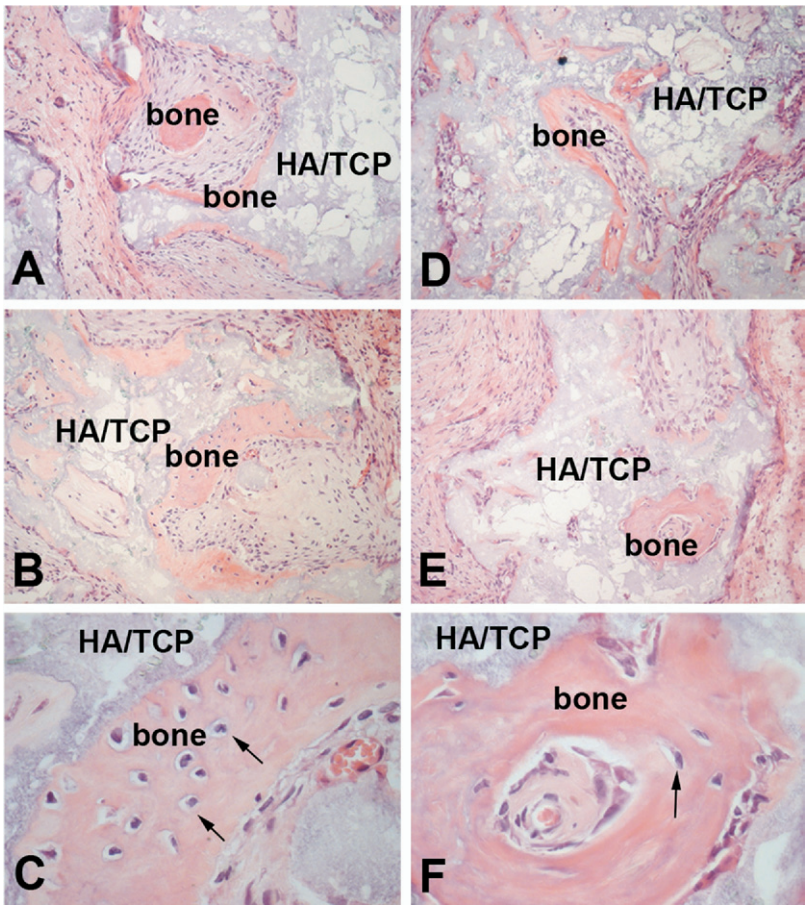

Fig. 7. In vivo osteogenic differentiation of maxillary/mandibular periosteal cells (**A-C**) and marrow stromal cells (**D-F**) expanded in dexamethasone-containing medium. Cells were loaded onto HA/TCP and transplanted into the subcutis of immunocompromised mice. Transplants were harvested after 8 weeks, decalcified, embedded in paraffin, and stained with hematoxylin and eosin. In all samples, transplanted cells differentiated into osteoblasts, deposited bone tissue, and transformed into mature osteocytes (**C** and **F**, *arrows*). (**A**, **B**, **D**, and **E**, original magnification ×50; **C** and **F**, original magnification ×100.)

stromal cells isolated from the iliac crest,[19,42] both bone marrow stromal cells and periosteal cells from the maxilla constitutively express the osteogenic transcription factor and master gene, *Runx2*. As in the case of iliac crest BMSCs, this property can be interpreted as a native commitment to the osteogenic lineage, and reflects an inherent ability to differentiate into mature bone-forming cells.[19,42] Indeed, when exposed to dexamethasone, which induces osteogenic differentiation in iliac crest BMSCs,[18] both M-PCs and MT-BMSCs responded with a marked increase in expression of the osteogenic marker alkaline phosphatase, as detected both by enzyme activity and FACS analysis.

Whereas in vitro assays and expression of individual or multiple osteogenic markers is a convenient, fast, and inexpensive empirical approach to probe the osteogenic capacity of a given cell strain in vitro, no in vitro assay demonstrates true osteogenic ability with the same stringency as an in vivo transplantation assay. We have shown that both M-PCs and MT-BMSCs can directly form histology-proven bone in vivo when transplanted into the subcutaneous tissue of immunocompromised mice. This directly demonstrates that cells isolated from the periosteum or the maxillary tuberosity can form bone in vivo following a phase of ex vivo expansion. Therefore, the results of our in vivo transplantation assay directly validate the biological properties of the cell strains under study as specifically osteogenic in vivo, and justify the design of a direct preclinical model for their use in the reconstruction of gnathic bones in the near future.

Because the goal of our study was to prove the inherent osteogenic ability of cell strains isolated from noncanonical sources, the carrier material chosen for our experiments had to be one with previously proven efficacy when used with osteogenic cells from canoni-

cal sources. The material we used, a combination of hydroxyapatite and tricalcium phosphate, was previously used in combination with iliac crest BMSCs in different in vivo studies.[18,43] These studies showed that the material is both osteoconductive and cell friendly. In the course of our study, we also tested a variety of additional commercial materials that are commonly used (without cells) for bone reconstruction and none proved satisfactory, pending further systematic experimentation. Meanwhile, we have shown that culture conditions directly influence the interaction of osteogenic cells with the material we used. Specifically, exposure to dexamethasone greatly increased the attachment of cultured cells to the biomaterial prior to in vivo transplantation. The biological effects of dexamethasone on cultured stromal cells are complex and poorly elucidated. Dexamethasone has mainly been seen as a (somewhat empirical) enhancer of the osteogenic differentiation potential. Our data identify the promotion of cell adhesion to an HA/TCP phase in vitro as an additional effect of dexamethasone, which has obvious direct bearing on the outcome of subsequent in vivo transplantation procedures. By increasing the number of cells retained in the cell-material construct to be transplanted, dexamethasone can influence in vivo bone formation in a manner dependent on its effects on cell adhesion and independent of its effects on cell differentiation. In general, this observation emphasizes the significance of culture conditions on the ultimate efficacy of a cell-biomaterial construct for bone tissue engineering.

In conclusion, our study indicates that the maxillary/mandibular periosteum and the maxillary bone marrow may effectively serve as reliable and easy-to-harvest intraoral sources of osteoprogenitor cells either for immediate generation of cell-scaffold constructs or for cell banking and subsequent repair of cranio-maxillofacial bone defects.

REFERENCES

1. Buser D, Hoffmann B, Bernard JP, Lussi A, Mettler D, Schenk RK. Evaluation of filling materials in membrane-protected bone defects. A comparative histomorphometric study in the mandible of miniature pigs. Clin Oral Implants Res 1998;9:137-50.
2. Buck BE, Resnick L, Shah SM, Malinin TI. Human immunodeficiency virus cultured from bone. Implications for transplantation. Clin Orthop Relat Res 1990;251:249-53.
3. Wenz B, Oesch B, Horst M. Analysis of the risk of transmitting bovine spongiform encephalopathy through bone grafts derived from bovine bone. Biomaterials 2001;12:1599-1606.
4. Schilling AF, Linhart W, Filke S, Gebauer M, Schinke T, Rueger JM, et al. Resorbability of bone substitute biomaterials by human osteoclasts. Biomaterials 2004;25:3963-72.
5. Velich N, Nemeth Z, Toth C, Szabo G. Long-term results with different bone substitutes used for sinus floor elevation. J Craniofac Surg 2004;15:38-41.
6. Nkenke E, Schultze-Mosgau S, Radespiel-Troger M, Kloss F, Neukam FW. Morbidity of harvesting of chin grafts: a prospective study. Clin Oral Impl Res 2001;12:495-502.
7. Ahlmann E, Patzakis M, Roidis N, Shepherd L, Holtom P. Comparison of anterior and posterior iliac crest bone grafts in terms of harvest-site morbidity and functional outcome. J Bone Joint Surg 2002;84A:716-20.
8. Coulthard P, Esposito M, Jokstad A, Worthington HV. Interventions for replacing missing teeth: bone augmentation techniques for dental implant treatment. Cochrane Database Syst Rev 2003;(3):CD003607.
9. St. John TA, Vaccaro AR, Sah AP, Schaefer M, Berta SC, Albert T, et al. Physical and monetary costs associated with autogenous bone graft harvesting. Am J Orthop 2003;32:18-23.
10. Hatano N, Shimizu Y, Ooya K. A clinical long-term radiographic evaluation of graft height changes after maxillary sinus floor augmentation with a 2:1 autogenous bone/xenograft mixture and simultaneous placement of dental implants. Clin Oral Impl Res 2004;15:339-45.
11. Kruyt MC, de Bruijn JD, Wilson CE, Oner FC, van Blitterswijk CA, Verbout AJ, et al. Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in goats. Tissue Eng 2003;9:327-36.
12. Kruyt MC, van Gaalen SM, Oner FC, Verbout AAJ, de Bruijn JD, Dhert WJ. Bone tissue engineering and spinal fusion: the potential of hybrid constructs by combining osteoprogenitor cells and scaffolds. Biomaterials 2004;25:1463-73.
13. Koole R, Bosker H, van der Dussen FN. Late secondary autogenous bone grafting in cleft patients comparing mandibular (ectomensenchymal) and iliac crest (mesenchymal) grafts. 1989;17(s1):28-30.
14. Bianco P, Riminucci M. [Stem cells in medicine]. Recenti Prog Med 2001;92:251-6. Italian.
15. Bianco P, Robey PG. Stem cells in tissue engineering. Nature 2001;414:118-21.
16. Gehron Robey P, Bianco P. The use of adult stem cells in rebuilding the human face. J Am Dent Assoc 2006;137:961-72.
17. Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Gehron Robey P. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 1997;63:1059-69.
18. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 1997;12:1335-47.
19. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest 2000;105:1663-8.
20. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001;19:180-92.
21. Cancedda R, Mastrogiacomo M, Bianchi G, Derubeis A, Muraglia A, Quarto R. Bone marrow stromal cells and their use in regenerating bone. Novartis Found Symp 2003;249:133-43.
22. Mankani MH, Kuznetsov SA, Shannon B, Nalla RK, Ritchie RO, Qin Y, et al. Canine cranial reconstruction using autologous bone marrow stromal cells. Am J Pathol 2006;168:542-50.
23. Mankani MH, Kuznetsov SA, Wolfe RM, Marshall GW, Robey PG. In vivo bone formation by human bone marrow stromal cells: reconstruction of the mouse calvarium and mandible. Stem Cells 2006;24:2140-9.
24. Mastrogiacomo M, Corsi A, Francioso E, Di Comite M, Monetti F, Scaglione S, et al. Reconstruction of extensive long bone defects in sheep using resorbable bioceramics based on silicon stabilized tricalcium phosphate. Tissue Eng 2006;12:1261-73.
25. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with

the use of autologous bone marrow stromal cells. New Engl J Med 2001;344:385-6.
26. Schmelzeisen R, Schimming R, Sittinger M. Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor augmentation—a preliminary report. J Craniomaxillofac Surg. 2003;31:34-9.
27. Schimming R, Schmelzeisen R. Tissue-engineered bone for maxillary sinus augmentation. J Oral Maxillofac Surg 2004;62:724-9.
28. Conejero JA, Lee JA, Parrett BM, Terry M, Wear-Maggitti K, Grant RT, et al. Repair of palatal bone defects using osteogenically differentiated fat-derived stem cells. Plast Reconstr Surg 2006;117:857-63.
29. Kuznetsov SA, Riminucci M, Gehron Robey P, Bianco P. Postnatal skeletal stem cells methods for isolation and analysis of bone marrow stromal cells (BMSCs) from post-natal murine and human marrow. In: Celis J, editor. Cell biology: a laboratory handbook. 3rd ed. San Diego: Elsevier; 2005. p. 79-86.
30. Kuznetsov SA, Friedenstein AJ, Gehron Robey P. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 1997;97:561-70.
31. Bianco P, Kuznetsov SA, Riminucci M, Gehron Robey P. Postnatal skeletal stem cells. Methods Enzymol 2006;419:117-48.
32. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2:83-92.
33. Gehron Robey P, Termine JD. Human bone cells in vitro. Calcif Tissue Int 1985;37:453-60.
34. Neckers LM, Trepel JB. Transferrin receptor expression and the control of cell growth. Cancer Invest 1986;4:461-70.
35. Larrick JW, Cresswell P. Modulation of cell surface iron transferrin receptors by cellular density and state of activation. J Supramol Struct 1979;11:579-86.
36. Bianco P, Gehron Robey P. Skeletal stem cells. In: Lanza HB RP, Melton DA, Moore MAS, Donnall Thomas (Hon) E, Verfaillie CM, Weissman IL, West MD, editors. Handbook of adult and fetal stem cells. Vol 2. New York: Academic Press; 415-24.
37. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 1988;136:42-60.
38. Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. Crit Rev Oral Biol Med 1999;10:165-81.
39. Akintoye SO, Lam T, Shi S, Brahim J, Collins MT, Robey PG. Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals. Bone 2006;38:758-68.
40. Matsubara T, Suardita K, Ishii M, Sugiyama M, Igarashi A, Oda R, et al. Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. J Bone Miner Res 2005;20:399-409.
41. De Bari C, Dell'Accio F, Vanlauwe J, Eyckmans J, Khan IM, Archer CW, et al. Mesenchymal multipotency of adult human periosteal cells demonstrated by single- cell lineage analysis. Arthritis Rheum 2006;54:1209-21.
42. Satomura K, Krebsbach P, Bianco P, Gehron Robey P. Osteogenic imprinting upstream of marrow stromal cell differentiation. J Cell Biochem 2000;78:391-403.
43. Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, et al. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000;49:328-37.

Reprint requests:

Professor Paolo Bianco
Dipartimento di Medicina Sperimentale e Patologia
Universita' La Sapienza
Viale Regina Elena 324
00161 Roma
p.bianco@flashnet.it